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(54) Title: DRUG DELIVERY COMPOSITIONS

(57) Abstract

A composition for administration to the mucosa comprises a pharmacologically active compound and a polycationic substance. The polycationic substance is preferably DEAE-dextran or chitosan and the pharmacologically active compound is preferably insulin or calcitonin. The composition may be a solution, dispersion, powder or microspheres. Other enhancers, such as lysophosphatidylcholine, can be included if desired.

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DRUG DELIVERY COMPOSITIONS

The present invention relates to drug delivery compositions and more particularly to compositions which provide for the uptake of active drug material across mucosal surfaces, such as the vagina, colon or the nasal cavity.

A major problem in drug delivery is the effective absorption of high molecular weight material such as proteins and peptides across biological membranes. Normally such molecules are not taken up by the body if administered to the gastrointestinal tract, the buccal mucosa, the rectal mucosa, the vaginal mucosa or the intranasal mucosa. Recent studies with insulin have demonstrated that the absorption of such a compound can be increased if it is given together with a so-called absorption enhancer. These absorption enhancing materials have included surfactants of the non-ionic type as well as various bile salt derivatives. An increased permeability of membranes in the presence of these types of surfactant materials is obtained and the literature in the field of gastroenterology contains a wide range of such absorption promoters. (For a review see Davis *et al* (editors), Delivery Systems for Peptide Drugs, Plenum Press, New York, 1987.) However, such materials will probably not be acceptable for the chronic administration of pharmacological agents because of their irritant effects on membranes. This includes not only the non-ionic variety of surface active agents but also bile salts and bile salt derivatives (e.g. fusidic acid).

EP-A-023 359 and EP-A-122 023 describe a powdery pharmaceutical composition for application to the nasal mucosa and methods for administration thereof. The pharmaceutical composition allows polypeptides and derivatives thereof to be effectively absorbed through the nasal mucosa. Similarly, US-A-4 226 848 describes a method for administering a powdery medicament to the nasal mucosa where the preferred composition has mucoadhesive properties.

EP-A-230 264 describes an aqueous nasal drug delivery system for vaccines containing a high molecular weight drug, a gelling agent (e.g. hydroxyethylcellulose) and in some cases other additives (e.g. surfactants, glycerol and polyethyleneglycol) but, again, the composition is administered as a powder.

Microsphere-containing formulations have been described in WO 88/09163. The formulations contain certain enhancers to aid effective penetration of the mucosa by the drug. Our co-pending application WO 89/03207 further describes formulations which do not require an enhancer. These formulations may comprise drug-containing microcapsules which are coated with DEAE-dextran.

DEAE-dextran has been proposed for use in oral drug delivery formulations, where it is believed to interact with gastro-intestinal mucins (Anderson, M.T. *et al*, oral presentation at a meeting of the Society for Experimental Biology, 24-29 July 1988, Manchester, U.K.) and has been delivered to the nasal

cavities of rabbits as a model compound to study the absorption of peptides of differing sizes (Maitani, Y., *et al*, Int. J. Pharm. 1989, 49, 23-27).

Igawa *et al* (1988 Chem. Pharm. Bull. 36(8) 3055-3059) administered human interferon-β intranasally to rabbits with a DEAE-dextran excipient. The dextran part of the latter had an average molecular weight of 9000 and did not enhance the absorption of the drug, and the authors concluded that low MW excipients were to be preferred to high MW components. In view of this, it is surprising to find, as we now have, that a solution or dispersion of relatively high MW DEAE-dextran or other polycationic substances such as chitosan can form the basis of an improved formulation which does not require other enhancers, although the presence of other enhancers may further improve the performance of the compositions.

Accordingly, one aspect of the invention provides a composition for administration to mucosa comprising a pharmacologically active compound and a polycationic substance, provided that the formulation does not comprise microcapsules coated with DEAE-dextran or, if for administration to gut mucosa, does not comprise a solution of DEAE-dextran.

The polycationic substance may be present as a solution in an aqueous medium, as a dispersion in an aqueous system, as a powder or as microspheres. Preferably, such microspheres are

formed from the polycationic substance itself (usually with the pharmacologically active substance incorporated as well) with or without other suitable microsphere-forming substances such as (human) serum albumin and derivatives and analogues thereof.

Preferably, the concentration of the polycationic substance in such a solution is 0.01 to 50% w/v, more preferably 0.1 to 50%, more preferably 0.2% to 30% and most preferably 0.5-15%.

Diethylaminoethyl-dextran (DEAE-dextran) is a polycationic derivative of dextran containing diethylaminoethyl groups coupled to the glucose residues by ether linkages. The parent dextran can have an average molecular weight of about 5,000 to 40 x 10^6, but is typically about 500,000. In the context of the present invention, the term is limited to dextran of MW 10000 or more. The nitrogen content is usually approximately 3.2% which corresponds to one charged group to three glucose units. "Tandem" groups, which are introduced as the result of side reactions, result in the presence of three different basic groups in approximately equal ratios.

Chitosan is deacetylated chitin, or poly-N-acetyl-D-glucosamine. It is available from Protan Laboratories Inc, Redmond, Washington 98052, USA and, depending on the grade selected, can be soluble in water up to pH 6.0. A 1% solution of non-water soluble chitosan (Sea Cure) may be made by making a slurry (eg 2g/100 ml) in water and adding an equal volume of organic acid

(eg 100 ml of 2% acetic acid) and stirring vigorously for one hour. Water-soluble chitosan (Sea Cure$^+$) may dissolve without organic or inorganic acids being present.

Chitosan has previously been used to precipitate proteinaceous material, to make surgical sutures and as an immunostimulant. It has also been employed previously in oral drug formulations in order to improve the dissolution of poorly soluble drugs (Sawayanagi *et al*, Chem. Pharm. Bull., 31, 2062-2068 (1983)) or for the sustained release of drugs (Nagai *et al*, Proc. Jt. US-Jpn. Semin. Adv. Chitin, Chitosan, Relat. Enzymes, 21-39. Zikakis J.P. (ed), Academic Press. Orlando (1984)) by a process of slow erosion from a hydrated compressed matrix.

DEAE-dextran and chitosan are preferred, but further polycationic substances which may be used in the compositions of the invention include other polycationic carbohydrates such as but not limited to inorganic or organic salts of chitosan and modified forms of chitosan (especially more positively charged ones), polyaminoacids such as polylysine, polyquaternary compounds, protamine, polyimine, DEAE-imine, polyvinylpyridine, polythiodiethylaminomethylethylene (P(TDAE)), polyhistidine, DEAE-methacrylate, DEAE-acrylamide, poly-p-aminostyrene, polyoxethane, co-polymethacrylates (e.g. copolymers of HPMA, N-(2-hydroxypropyl)-methacrylamide), GAFQUAT (US Pat No 3,910,862) and polyamidoamines. The polycationic substances used in the invention have a molecular weight of 10 000 or more, preferably

at least 100 000 or 200 000 and most preferably about 500 000. The chitosan (or a salt thereof) preferably has an intrinsic viscosity of at least 400 ml/g, more preferably at least 500, 750 or 1000 ml/g.

If desired, other enhancers may be included in the compositions of the invention, for example lysophosphatidylcholine and generally all those mentioned in WO 88/09163. Gelling agents or viscosity-increasing substances may be added in order to help retain the formulation on the mucosa. The chitosan, in particular, may be formulated as microspheres with or without albumin.

The compositions may be prepared at a neutral pH, i.e. pH 6.5-7.5, preferably about 7.3, for example using a standard phosphate buffer or at lower pH, for example pH4, by addition of HCl to the above or by use of an alternative buffer system. However, it has been found that DEAE-dextran or chitosan in combination with at least some drugs, for example insulin and most if not all other proteins, form a complex. At lower or higher pH's, i.e. away from the isoelectric point of the polycation and the drug, this complex may be present as a true solution instead of a dispersion. This may be advantageous, although it is also the case that very low pH's are more likely to irritate or even harm the mucosa. Thus, the man skilled in

the art will be able to determine the optimal pH, which may lie between 1.0 and 11.0, preferably 4.0 to 7.5, for example 4.0 to 6.0, or 9.0 to 11.0.

The said complex may be isolated. The complex and its therapeutic utilities form further aspects of the invention.

The term "pharmacologically active compound" includes drugs, vaccines and components thereof (for example isolated antigens or parts thereof) and monoclonal antibodies.

The compositions may be used with drugs selected from the following non-exclusive list: insulin, calcitonins (for example porcine, human, salmon, chicken or eel) and synthetic modifications thereof, enkephalins, LHRH and analogues (Nafarelin, Buserelin, Zolidex), GHRH (growth hormone releasing hormone), nifedipin, THF(thymic humoral factor), CGRP (calcitonin gene related peptide), atrial natriuretic peptide, antibiotics, metoclopramide, ergotamine, Pizotizin, nasal vaccines (particularly AIDS vaccines, measles, rhinovirus Type 13 and respiratory syncitial virus), pentamidine and CCK (cholecystykinin).

Further drugs include: antibiotics and antimicrobial agents such as tetracycline hydrochloride, leucomycin, penicillin, penicillin derivatives, erythromycin, sulphathiazole and nitrofurazone; local anaesthetics such as benzocaine;

vasoconstrictors such as phenylephrine hydrochloride, tetrahydrozoline hydrochloride, naphazoline nitrate, oxymetazoline hydrochloride and tramazoline hydrochloride; cardiotonics such as digitalis and digoxin; vasodilators such as nitro-glycerine and papaverine hydrochloride; antiseptics such as chlorhexidine hydrochloride, hexylresorcinol, dequaliniumchloride and ethacridine; enzymes such as lysozyme chloride, dextranase; bone metabolism controlling agents such as vitamin D, and active vitamin D_3; sex hormones; hypotensives; sedatives; anti-tumor agents; steroidal anti-inflammatory agents such as hydro-cortisone, prednisone, fluticasone, prednisolone, triamcinolone, triamcinolone acetonide, dexamethasone, betamethasone, beclomethasone, and beclomethasone dipropionate; non-steroidal anti-inflammatory agents such as acetaminophen, aspirin, aminopyrine, phenylbutazone, mefanamic acid, ibuprofen, diclofenac sodium, indomethacin, colchicine, and probenocid; enzymatic anti-inflammatory agents such as chymotrypsin and bromelain seratiopeptidase; anti-histaminic agents such as diphenhydramine hydrochloride, chloropheniramine maleate and clemastine; and anti-allergic agents and antitussive-expectorant antiasthmatic agents such as sodium chromoglycate, codeine phosphate, and isoproterenol hydrochloride.

The compositions can be administered via the nasal route using a nasal spray device, pressurized aerosol cannister or simple instillation means. The compositions may gel on the mucosa, at least to some extent, and this may facilitate retention of the

composition on the mucosa. Formulations suitable for delivery of drugs to the colon can be subdivided into a number of technical categories known to those skilled in the art of pharmaceutical formulation. These can utilise coated solid dosage forms, such as tablets, pellets, mini-tablets, hard gelatin capsules etc or coated semi-solid preparations, such as soft gelatin capsules and the like. Enteric coated systems, based for example on methacrylate copolymers such as Eudragit L (Poly (methacrylic acid, methyl methacrylate)), are only soluble at pH 6 and above, so that the polymer only begins to dissolve on entry into the small intestine. The site of the disintegration is then dependent on the rate of intestinal transit and the amount of polymer present, a relatively thick polymer coating having been defined for delivery to the proximal colon (Hardy *et al*, Aliment. Pharmacol. Therap., 1, 273-280, (1987)). Polymers capable of providing site-specific colonic delivery can be utilised. These typically rely on the bacterial flora of the large bowel to provide enzymatic degradation of the polymer coat and hence release of the drug. A number of candidate materials appear promising, such as the azopolymers (Saffran *et al*, US Patent 4,663,308), glycosides (Friend *et al*, J. Med. Chem., 27, 261-266, (1984)) and a variety of naturally available and modified polysaccharides (Archer & Ring PCT Application GB89/00581).

Novel pulsed release technology (Magruder *et al*, US Patent 4,777,049) and the like, which permits drug delivery at a pre-determined time, is now available. Such systems can be used to deliver both drug and polycationic substance, together with other additives that may alter the local microenvironment to promote drug stability and uptake, directly to the colon and do not rely on external conditions to provide *in vivo* release, except for the presence of water.

A further aspect of the invention provides a method of treating a human or other mammal by administering a composition as described above to a mucosal surface of that human or other mammal, for example the vagina, eye, colon or nasal cavity.

Embodiments of the present invention will now be described by way of example.

EXAMPLE 1 : INSULIN PLUS DEAE-DEXTRAN

A rat *in vivo* experimental model, modified from that originally described by Hirai *et al* (1981 Int. J. Pharm., 7 317-325) and Fisher *et al* (1987 J. Pharm. Pharmacol., 39 357-362), was used to study the intranasal absorption of insulin aqueous solutions. Male Wistar rats (Bantin and Kingman) of approximate weight 200-250g, fasted overnight for about 20 hours, are anaesthetised by i.p. injection of 80 mg/kg pentobarbitone sodium (60 mg/ml Sagatal (Regd. T.M.) May and Baker) with further i.p. injections

of 0.05 ml when necessary to maintain a suitable level of anaesthesia. The rats are tracheotomized, the oesophagus sealed and the carotid artery and jugular vein cannulated.

Insulin (semisynthetic human Na-insulin) solutions were prepared in 1/75 M phosphate buffer of pH 7.3 to give a concentration of 167 IU/ml and the DEAE-dextran added to give concentrations of 10% w/v, 5% w/v or 1% w/v. The DEAE-dextran used in these experiments has a molecular weight of 500 000.

It is also possible to make up a solution of 334 IU/ml of insulin in phosphate buffer and add equal volumes of the DEAE-dextran in phosphate buffer of 20, 10 or 2% strength. This will give the same end solutions. When mixing the insulin solution with the DEAE-dextran the solution becomes turbid indicating that an interaction between the insulin and the DEAE-dextran has taken place.

An insulin solution containing the Laureth-9 enhancer system was prepared in a similar way.

The insulin solution alone or the insulin solutions containing the Laureth-9 or the various concentrations of DEAE-dextran were administered nasally to rats (n=4) at 16.7 IU/kg bodyweight using a Hamilton microsyringe. A volume of 20 µl was administered.

Blood samples of 0.2 ml were collected in Fluoride oxalate tubes from the carotid artery at 10 and 5 min. prior to the insulin administration and at 5, 15, 30, 45, 60, 90, 120, 180, 240 and 300 min. post-administration. The samples were kept for a short time on crushed ice until analysed on a Yellow Springs 23 AM glucose analyser by the glucose oxidase method.

Table 1 shows the approximate glucose levels (mmol/l) of rats given a dose of insulin in phosphate buffer and doses of insulin in phosphate buffer (pH 7.3) containing 1%, 5% or 10% DEAE-dextran measured at 120 minutes after administration. The level at the time of administration was about 3.5-4.0 mmol/l. The results show that insulin given intranasally as a simple phosphate buffer solution (pH 7.3) does not significantly lower the blood glucose level whereas the addition of the DEAE-dextran causes fast and significant decreases in blood glucose levels. The effect increases with increasing concentration of DEAE-dextran. The rats given the 10% concentration died early of hypoglycaemia. Administration of phosphate buffer alone shows a similar trend to that of the insulin solution alone, i.e. an increase in plasma glucose from about 3.5-4.0 mmol/l to about 5 mmol/l.

Table 1

	Blood glucose level (mmol/l)
Insulin plus DEAE-dextran 1%	1.6
Insulin plus DEAE-dextran 5%	1.2
Insulin plus DEAE-dextran 10%	1.0
Insulin alone	5.1

For comparison, the glucose levels of rats given a dose of insulin in phosphate buffer and rats given a dose of insulin in phosphate buffer containing 0.5% Laureth-9 show that this well known effective enhancer system gives a decrease in blood glucose concentration similar to the 1% DEAE-dextran (about 1.9 mmol/l at 120 mins).

Example 2 : EFFECT OF pH ON INSULIN/DEAE-DEXTRAN SOLUTIONS

Solutions containing DEAE-Dextran 1% w/v and Na-Insulin 167 IU/ml were prepared, separately and combined, in phosphate buffer (pH 7.3) and their pH measured using a Gallenkamp pH Stick. The appearance of each solution was noted. The effect of addition of 1M sodium hydroxide solution (NaOH) or 0.1M

hydrochloric acid (HCl) was determined. The two separate solutions were each clear (DEAE-D pH 6.58; Insulin pH 7.38) whereas the mixture (pH 6.65) was turbid.

The addition of 0.1M HCl to solutions of DEAE-dextran alone had no effect on solution appearance which remained clear. Solutions of Na-insulin however, became turbid when the pH reached 6.65 but cleared after further addition of acid lowered the pH to 4.14. Solutions of DEAE-dextran combined with Na-Insulin became less turbid after the addition of acid and were clear at pH 4.14. The addition of 1.0M NaOH to solutions of DEAE-dextran and Na-insulin alone had no effect on solution appearance which remained clear. Combined solutions of DEAE-dextran and Na-insulin however became less turbid as the pH increased and formed a clear solution when the pH reached 9.32. Solutions of DEAE-dextran and Na-insulin at about pH 4.0 were found to be at least as effective as those at about pH 6.6 in the rat model described above.

EXAMPLE 3 : TOXICITY OF A COMPOSITION OF THE INVENTION

Insulin 100IU/ml with DEAE-Dextran 5%w/v

The effects of the DEAE-dextran formulation on the nasal mucosa in rats (after 60 min incubation) were less dramatic than those of prior art surfactant enhancers. A few cells lost from the septum and turbinates were visible and mucus discharge on the

dosed side resulted in a slight decrease in epithelium height. The clear cell structure was not so well defined and cytoplasmic space appeared reduced. The epithelium still appeared to be more than one cell thick (i.e. pseudostratified) and formed a continuous layer, though the arrangement of nuclei above the basement membrane was altered. Cilia were not always distinct amongst the discharged mucus.

Considerable amounts of AB staining mucus were still apparent in cells on the dosed side though there was generally not the confluent spread of filled goblet cells as on the undosed side. Some mucus was again present in the undosed cavity of some animals.

Effects of this formulation were generally restricted to the ventral half of the cavity and lateral nasoturbinate i.e. the dorsal meatus was unaffected.

EXAMPLE 4 : (COMPARATIVE EXAMPLE) TOXICITY OF PRIOR ART COMPOSITION

Insulin 100IU/ml with STDHF 1%w/v

As compared to DEAE-dextran 5% w/v, STDHF (sodiumtaurodihydroxyfusidate) administered in the same way to rats and incubated for 60 mins showed obvious disruption to the nasal epithelium. Large volumes of mucus were apparent together

with cell loss, epithelium rearrangement and considerable reduction of epithelium height to about half that on the undosed side. Generally the full length of the dosed septum and turbinates were affected. AB staining showed that some mucus remained in many of the epithelial cells but others had discharged their whole mucus content, particularly where the epithelium was reduced to a thin single cell layer such as in the middle meatus.

Some mucus was apparent on the undosed septum or drained into the dorsal meatus, but with no cell loss. The undosed turbinates were unaffected. Epithelial height on the dosed side was consistently less than that on the undosed 'control' side.

EXAMPLE 5 : INSULIN PLUS CHITOSAN IN THE RAT

This Example was performed to evaluate the effect of chitosan, low or medium viscosity water soluble formulations (Sea cure^{+}), at different concentrations and at pH values of 4 and 7.3-7.4 on the intranasal absorption of insulin in rats (n=4).

Semisynthetic Na-insulin and chitosan (Sea cure^{+}) (water soluble powder) low viscosity (l.v.)and medium viscosity (m.v) from Protan Laboratories Inc. were used.

All insulin solutions were initially made in 14.65 mM phosphate buffer of pH 7.3-7.4 prepared from 1.904 g/l $Na_2HPO_4.2H_2O$ and 0.616 g/l $NaH_2PO_4.2H_2O$ in double distilled water. Adjustment of the pH to 4 where necessary was made by the addition of 150 µl of 0.1M HCl per ml of solution. Each 1 mg of insulin was considered equivalent to 28IU. Double-strength insulin stock solutions were prepared freshly as follows: 159.9 IU/ml (6.74 mg/ml) for administration at pH 7.3-7.4 and 183.8 IU/ml (7.75 mg/ml) for administration at pH 4, accounting for the dilution by the addition of 0.1M HCl. The expected water content of the insulin is 15.3%.

Double strength chitosan solutions were prepared as follows: 0.2% w/v l.v. (2 mg/ml) for use at pH 7.3-7.4; 1.0% w/v l.v. (10 mg/ml) for use at pH 7.3-7.4; 0.2% w/v l.v. (2.3 mg/ml) for use at pH 4; 1.0% w/v l.v. (11.5 mg/ml) for use at pH 4; and 0.2% w/v m.v. (2.3 mg/ml) for use at pH 4.

Insulin/chitosan formulations were prepared by mixing equi-volumes of the appropriate stock insulin and chitosan solutions and the addition of 150µl/ml of 0.1M HCl where necessary. Solutions were administered intranasally to rats at 100µl/kg, corresponding to doses of 8 IU/kg insulin with 0.1 or 0.5 mg/kg l.v. chitosan or 0.1 mg/kg m.v. chitosan. A dose of 100 µl/kg of Insulin (167 IU/ml) is instilled into the nasal cavity via a microsyringe (Hamilton) and 0.61mm o.d. polypropylene tubing (Portex).

Blood samples of 150 µl (8-12 drops) were collected from the carotid artery in fluoride oxalate blood tubes at 10, 6 and 2 minutes pre-administration and 5, 10, 15, 20, 40, 60, 90, 120, 180 and 240 minutes post-administration. Fluid replacement was given in the form of 0.9% saline via the jugular vein. The glucose levels of the samples were assayed within 2 hours of being taken using the glucose oxidase method on a Yellow Springs 23AM glucose analyser.

The pH 4 solutions were not buffered systems. A suitable buffered system may be devised if desirable.

All of the formulations gave a rapid fall in blood glucose levels, the 0.5% l.v. pH 4.0 solution reducing the level from 100% to about 16% after 60 minutes. Generally, 0.5% material was more effective than 0.1% and pH 4.0 was better than pH 7.3-7.4.

EXAMPLE 6 : INSULIN PLUS CHITOSAN IN THE SHEEP

Semi-synthetic human Na-insulin supplied by Nordisk, Gentofte was used. The water content of the sample was determined by spectrophotometry to be approximately 15%. Chitosan SEA CURE+, which is water soluble, of low (intrinsic viscosity 388 ml/g) and medium viscosity (intrinsic viscosity 1010 ml/g) were obtained from Protan Laboratories Inc. These will be referred to as CSN LV and CSN MV, respectively. Sixteen cross-bred sheep

of known weight were used. The animals were not fasted prior to insulin administration. An in-dwelling Viggo secalon cannula of 1.2 mm i.d., fitted with a secalon universal flow-switch, was placed approx. 15 cm into one of the external jugular veins of each animal on the first day of the study and, whenever necessary, was kept patent by flushing it with heparinised normal saline (25 IU/ml). This cannula was removed upon the completion of the study.

An insulin solution of 19.32 mg/ml (460 IU/ml) was prepared in 14.65 mM phosphate buffer (0.476g $Na_2HPO_4.2H_2O$+0.154g $Na_2PO_4.2H_2O$ in 250 ml water) of pH 7.3-7.4, and filtered on a 0.2 µm membrane filter (Corning 21052-25). Chitosan solutions were prepared in 14.65 mM phosphate buffer as follows: 2.3 mg/ml CSN LV, 11.5 mg/ml CSN LV, 2.3mg/ml CSN MV or 11.5 mg/ml CSN MV. Insulin/chitosan formulations were produced by mixing equal volumes of the insulin stock solution and the appropriate chitosan solution, followed by the addition of 0.15 ml of 0.166 M hydrochloric acid for each 1.0 ml of the mixture. The addition of hydrochloric acid proved necessary to ensure that the chitosan remained in solution.

The final formulations thus produced had the following composition:

Formulation 1: 200 IU/ml insulin + 0.1% CSN LV, pH 3.6

Formulation 2: 200 IU/ml insulin + 0.5% CSN LV, pH 4.4

Formulation 3: 200 IU/ml insulin + 0.1% CSN MV, pH 3.6

Formulation 4: 200 IU/ml insulin + 0.5% CSN MV, pH 4.4

The sheep were divided into 4 groups, each of 3 animals, with each sheep receiving 2.0 IU/kg insulin intranasally in the form of an aqueous solution of Formulation 1, 2, 3 or 4, corresponding to Groups 1 to 4.

For the intranasal studies, the sheep were sedated by use of an i.v. dose of ketamine hydrochloride at 2.25 mg/kg. This was intended as a counter-measure against the animal sneezing during administration. The anaesthesia lasted for about 3 minutes. Blood samples of 6 ml were collected onto crushed ice from the cannulated jugular vein of the sheep at 15 and 5 min prior to the insulin administration and at various times post-administration. Each blood sample was divided into two parts. For insulin analysis, the blood collected (4.0 ml) was mixed gently in 5 ml heparinised (Li Heparin) tubes. For glucose analysis, the blood collected (2.0 ml) was mixed gently in 5 ml fluoride oxalate tubes. The plasma was separated by centrifugation at 4°C and 3000 rpm, and then stored at -20°C awaiting insulin and glucose analysis.

The following results were obtained:

Table 2

	Mean blood glucose level (mmol/l)	
	5 mins post-administration	75 mins post-administration
Group 1	3.4	3.0
Group 2	3.4	2.5
Group 3	3.4	2.6
Group 4	3.8	1.8

CLAIMS

1. A composition for administration to mucosa comprising a pharmacologically active compound and a polycationic substance provided that the composition does not comprise microcapsules coated with DEAE-dextran or, if for administration to gut mucosa, does not comprise a solution of DEAE-dextran.

2. A composition according to Claim 1 wherein the composition comprises microspheres of the polycationic substance.

3. A composition according to Claim 1 wherein the composition comprises a solution or dispersion of the polycationic substance.

4. A composition according to Claim 3 wherein the concentration of the polycationic substance is 0.5-15% w/v.

5. A composition according to any other of the preceding claims wherein the pharmacologically active compound is insulin.

6. A composition according to any one of the preceding claims wherein the polycationic substance is DEAE-dextran or chitosan or a salt or derivative thereof.

7. A method of delivering a pharmacologically active compound to a human or other mammal comprising administering a composition according to any one of the preceding claims to the mucosa of the human or other mammal.

8. A complex of a pharmacologically active compound and a polycationic substance.

9. A complex according to Claim 8 for therapeutic use.

10. The use of a complex according to Claim 8 in the manufacture of a medicament for delivery to a mucosal surface.

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 90/00291

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[5]: A 61 K 9/06, A 61 K 47/36

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[5]	A 61 K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	DE, A, 3200766 (TOKYO JOZO CO. LTD) 16 September 1982 see claims 1,6; page 22, lines 12-14 --	1,6
X	Biological Abstracts, volume 30, no. 11, Y. Sawayanagi et al.: "Directly compressed tablets containing chitin or chitosan in addition to mannitol", pages 4216-4218 see abstract 75074688 --	1,6
X,P	Chemical Abstracts, volume 112, 1990, (Columbus, Ohio, US), A. Manosroi et al.: "The entrapment of a human insulin-DEAE dextran complex in different compound liposomes", see abstract 84024c & Drug Dev. Ind. Pharm. 1989, 15(14-16), 2531-46	1,2,5,6, 8-10

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

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IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
25th May 1990	29. 06. 90
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	H. Daniels — H. DANIELS

Form PCT/ISA/210 (second sheet) (January 1985)

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. [X] OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This International search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. [X] Claim numbers ....8...., because they relate to subject matter not required to be searched by this Authority, namely:

See PCT Rule 39.1(iv)
Methods for treatment of the human or animal body by means of surgery or therapy, as well as diagnostic methods.

2. [ ] Claim numbers....., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claim numbers....., because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. [ ] OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
3. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
4. [ ] As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

- [ ] The additional search fees were accompanied by applicant's protest.
- [ ] No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. GB 9000291

SA 34698

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 26/06/90
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
DE-A- 3200766	16-09-82	JP-A,B 57118509	23-07-82
		JP-A- 57146722	10-09-82
		JP-A- 57146723	10-09-82
		JP-A- 58085813	23-05-83
		CA-A- 1184492	26-03-85
		DE-A- 3250023	29-03-90
		FR-A,B 2497669	16-07-82
		GB-A,B 2092002	11-08-82
		US-A- 4900730	13-02-90
		JP-A- 57135033	20-08-82
		SE-A- 8200103	15-07-82
		US-A- 4609640	02-09-86

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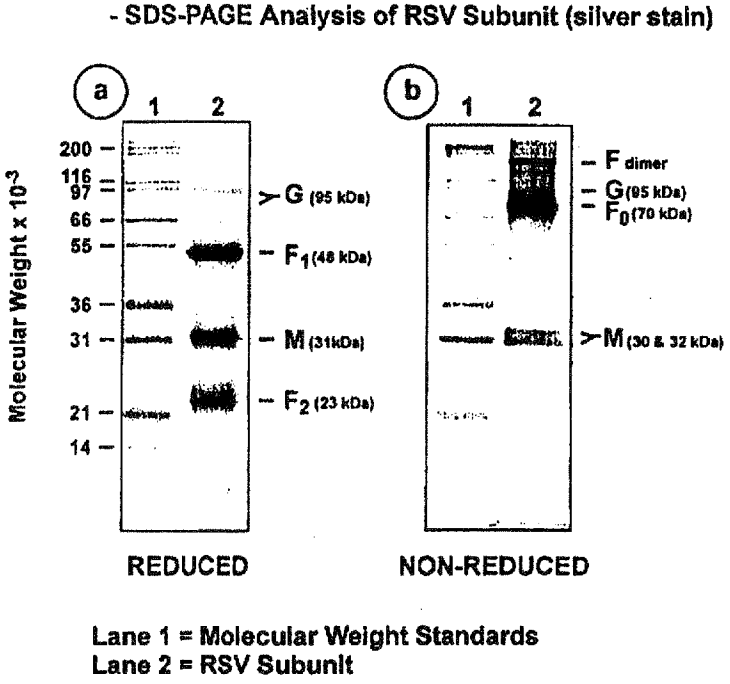
(54) Title: SUBUNIT RESPIRATORY SYNCYTIAL VIRUS VACCINE PREPARATION

(57) Abstract

The fusion (F) protein, attachment (G) protein and matrix (M) protein of respiratory syncytial virus (RSV) are isolated and purified from respiratory syncytial virus by mild detergent extraction of the proteins from concentrated virus, loading the protein onto a hydroxyapatide or other ion-exchange matrix column and eluting the protein using mild salt treatment. The F, G and M proteins, formulated as immunogenic compositions, are safe and highly immunogenic and protect relevant animal models against disease caused by respiratory syncytial virus infection.



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TITLE OF INVENTION

SUBUNIT RESPIRATORY SYNCYTIAL VIRUS VACCINE PREPARATION

FIELD OF INVENTION

The present invention is related to the field of immunology and is particularly concerned with vaccine preparations against respiratory syncytial virus infection.

REFERENCE TO RELATED APPLICATION

This application is a continuation-in-part of copending United States Patent Application No. 08/679,060 filed July 12, 1996.

BACKGROUND OF THE INVENTION

Human respiratory syncytial virus is the main cause of lower respiratory tract infections among infants and young children (refs. 1 to 3 - a list of references appears at the end of the disclosure and each of the references in the list is incorporated herein by reference thereto). Globally, 65 million infections occur every year resulting in 160,000 deaths (ref. 4). In the USA alone 100,000 children may require hospitalization for pneumonia and bronchiolitis caused by RS virus in a single year (refs. 5, 6). Providing inpatient and ambulatory care for children with RS virus infections costs in excess of $340 million annually in the USA (ref. 7). Severe lower respiratory tract disease due to RS virus infection predominantly occurs in infants two to six months of age (ref. 8). Approximately 4,000 infants in the USA die each year from complications arising from severe respiratory tract disease caused by infection with RS virus and Parainfluenza type 3 virus (PIV-3). The World Health Organization (WHO) and the National Institute of Allergy and Infectious Disease (NIAID) vaccine advisory

committees have ranked RS virus second only to HIV for vaccine development.

The structure and composition of RSV has been elucidated and is described in detail in the textbook "Fields Virology", Fields, B.N. et al. Raven Press, N.Y. (1996), in particular, Chapter 44, pp 1313-1351 "Respiratory Syncytial Virus" by Collins, P., McIntosh, K., and Chanock, R.M. (ref. 9).

The two major protective antigens of RSV are the envelope fusion (F) and attachment (G) glycoproteins (ref. 10). The F protein is synthesized as an about 68 kDa precursor molecule (F_0) which is proteolytically cleaved into disulfide-linked F_1 (about 48 kDa) and F_2 (about 20 kDa) polypeptide fragments (ref. 11). The G protein (about 33 kDa) is heavily O-glycosylated giving rise to a glycoprotein of apparent molecular weight of about 90 kDa (ref. 12). Two broad subtypes of RS virus have been defined A and B (ref. 13). The major antigenic differences between these subtypes are found in the G glycoprotein while the F glycoprotein is more conserved (refs. 7, 14).

In addition to the antibody response generated by the F and G glycoproteins, human cytotoxic T cells produced by RSV infection have been shown to recognize the RSV F protein, matrix protein M, nucleoprotein N, small hydrophobic protein SH, and the nonstructural protein 1b (ref. 15).

A safe and effective RSV vaccine is not available and is urgently needed. Approaches to the development of RS virus vaccines have included inactivation of the virus with formalin (ref. 16), isolation of cold-adapted and/or temperature-sensitive mutant viruses (ref. 17) and purified F or G glycoproteins (refs. 18, 19, 20). Clinical trial results have shown that both live attenuated and formalin-inactivated vaccines failed to adequately protect vaccines against RS virus infection

(refs. 21 to 23). Problems encountered with attenuated cold-adapted and/or temperature-sensitive RS virus mutants administered intranasally included clinical morbidity, genetic instability and overattenuation (refs. 24 to 26). A live RS virus vaccine administered subcutaneously also was not efficacious (ref. 27). Inactivated RS viral vaccines have typically been prepared using formaldehyde as the inactivating agent. Murphy et al. (ref. 28) have reported data on the immune response in infants and children immunized with formalin-inactivated RS virus. Infants (2 to 6 months of age) developed a high titre of antibodies to the F glycoprotein but had a poor response to the G protein. Older individuals (7 to 40 months of age) developed titres of F and G antibodies comparable to those in children who were infected with RS virus. However, both infants and children developed a lower level of neutralizing antibodies than did individuals of comparable age with natural RS virus infections. The unbalanced immune response, with high titres of antibodies to the main immunogenic RS virus proteins F (fusion) and G (attachment) proteins but a low neutralizing antibody titre, may be in part due to alterations of important epitopes in the F and G glycoproteins by the formalin treatment. Furthermore, some infants who received the formalin-inactivated RS virus vaccine developed a more serious lower respiratory tract disease following subsequent exposure to natural RS virus than did non-immunized individuals (refs. 22, 23). The formalin-inactivated RS virus vaccines, therefore, have been deemed unacceptable for human use.

Evidence of an aberrant immune response also was seen in cotton rats immunized with formalin-inactivated RS virus (ref. 29). Furthermore, evaluation of RS virus formalin-inactivated vaccine in cotton rats also

showed that upon live virus challenge, immunized animals developed enhanced pulmonary histopathology (ref. 30).

The mechanism of disease potentiation caused by formalin-inactivated RS virus vaccine preparations remains to be defined but is a major obstacle in the development of an effective RS virus vaccine. The potentiation may be partly due to the action of formalin on the F and G glycoproteins. Additionally, a non-RS virus specific mechanism of disease potentiation has been suggested, in which an immunological response to contaminating cellular or serum components present in the vaccine preparation could contribute, in part, to the exacerbated disease (ref. 31). Indeed, mice and cotton rats vaccinated with a lysate of HEp-2 cells and challenged with RS virus grown on HEp-2 cells developed a heightened pulmonary inflammatory response.

Furthermore, RS virus glycoproteins purified by immunoaffinity chromatography using elution at acid pH were immunogenic and protective but also induced immunopotentiation in cotton rats (refs. 29, 32).

There clearly remains a need for immunogenic preparations, including vaccines, which are not only effective in conferring protection against disease caused by RSV but also do not produce unwanted side-effects, such as immunopotentiation. There is also a need for antigens for diagnosing RSV infection and immunogens for the generation of antibodies (including monoclonal antibodies) that specifically recognize RSV proteins for use, for example, in diagnosis of disease caused by RS virus.

SUMMARY OF THE INVENTION

The present invention provides the production of respiratory syncytial virus (RSV) on a vaccine quality cell line, for example, VERO, MRC5 or WI38 cells, purification of the virus from fermentor harvests, extraction of the F, G and M proteins from the purified

virus and copurification of the F, G and M proteins without involving immunoaffinity or lentil lectin or concanavalin A affinity steps. In particular, the lectin affinity procedure, described, for example, in WO 91/00104 (US 07/773,949 filed June 28, 1990) assigned to the assignee hereof and the disclosure of which is incorporated herein by reference), could lead to leaching of the ligand into the product.

In addition, there is provided herein, for the first time, a procedure for the coisolation and copurification of the F, G and M proteins of RSV and also immunogenic compositions comprising copurified mixtures of the RSV proteins.

The coisolated and copurified F, G and M RSV proteins are non-pyrogenic, non-immunopotentiating, and substantially free of serum and cellular contaminants. The isolated and purified proteins are immunogenic, free of any infectious RSV and other adventitious agents.

Accordingly, in one aspect of the present invention, there is provided a mixture of purified fusion (F) protein, attachment (G) protein and matrix (M) protein of respiratory syncytial virus (RSV).

The fusion (F) protein may comprise multimeric fusion (F) proteins, which may include, when analyzed under non-reducing conditions, heterodimers of molecular weight approximately 70 kDa and dimeric and trimeric forms.

The attachment (G) protein may comprise, when analyzed under non-reducing conditions, oligomeric G protein, G protein of molecular weight approximately 95 kDa and G protein of molecular weight approximately 55 kDa.

The matrix (M) protein may comprise, when analyzed under non-reducing conditions, protein of molecular weight approximately 28 to 34 kDa.

The protein mixture provided herein, when analyzed by reduced SDS-PAGE analysis, may comprise the fusion (F) protein comprising F_1 of molecular weight approximately 48 kDa and F_2 of about 23 kDa, the attachment (G) protein comprising a G protein of molecular weight approximately 95 kDa and a G protein of molecular weight approximately 55 kDa, and the matrix (M) protein comprising an M protein of approximately 31 kDa.

The mixture provided in accordance with this aspect of the invention may comprise the F, G and M proteins in the relative proportions of:

F about 35 to about 70 wt%
G about 5 to about 30 wt%
M about 10 to about 40 wt%

When analyzed by SDS-PAGE under reducing conditions and densitometric scanning following silver staining, the ratio of F_1 of molecular weight approximately 48 kDa to F_2 of molecular weight approximately 23 kDa in this mixture may be approximately between 1:1 and 2:1. The mixture of F, G and M proteins may have a purity of at least about 75%, preferably at least about 85%.

The mixture provided herein in accordance with this aspect of the invention, having regard to the method of isolation employed herein as described below, is devoid of monoclonal antibodies and devoid of lentil lectin and concanavalin A.

The RSV proteins provided in the mixture of proteins provided herein generally are substantially non-denatured by the mild conditions of preparation and may comprise RSV proteins from one or both of subtypes RSV A and RSV B.

In accordance with a preferred embodiment of the invention, there is provided a coisolated and copurified mixture of non-denatured proteins of respiratory syncytial virus (RSV), consisting essentially of the

fusion (F) protein, attachment (G) protein and matrix (M) protein of RSV, wherein the mixture is free from lentil-lectins including concanavalin A and from monoclonal antibodies.

In accordance with another aspect of the present invention, there is provided an immunogenic preparation comprising an immunoeffective amount of the mixtures provided herein.

The immunogenic compositions provided herein may be formulated as a vaccine containing the F, G and M proteins for *in vivo* administration to a host, which may be a primate, specifically a human host, to confer protection against disease caused by RSV.

The immunogenic compositions of the invention may be formulated as microparticles, capsules, ISCOMs or liposomes. The immunogenic compositions may further comprise at least one other immunogenic or immunostimulating material, which may be at least one adjuvant or at least one immunomodulator, such as cytokines including ILK.

The at least one adjuvant may be selected from the group consisting of aluminum phosphate, aluminum hydroxide, QS21, Quil A or derivatives or components thereof, calcium phosphate, calcium hydroxide, zinc hydroxide, a glycolipid analog, an octodecyl ester of an amino acid, a muramyl dipeptide, polyphosphazene, a lipoprotein, ISCOM matrix, DC-Chol, DDA, and other adjuvants and bacterial toxins, components and derivatives thereof as, for example, described in USSN 08/258,228 filed June 10, 1994, assigned to the assignee hereof and the disclosure of which is incorporated herein by reference thereto (WO 95/34323). Under particular circumstances, adjuvants that induce a Th1 response are desirable.

The immunogenic compositions provided herein may be formulated to comprise at least one additional

immunogen, which conveniently may comprise a human parainfluenza virus (PIV) protein from PIV-1, PIV-2 and/or PIV-3, such as the PIV F and HN proteins. However, other immunogens, such as from *Chlamydia*, polio, hepatitis B, diphtheria toxoid, tetanus toxoid, influenza, haemophilus, *B. pertussis*, pneumococci, mycobacteria, hepatitis A and *Moraxella* also may be incorporated into the compositions, as polyvalent (combination) vaccines.

An additional aspect of the present invention provides a method of generating an immune response in a host by administering thereto an immunoeffective amount of the immunogenic composition provided herein. Preferably, the immunogenic composition is formulated as a vaccine for *in vivo* administration to the host and the administration to the host, including humans, confers protection against disease caused by RSV. The immune response may be humoral or a cell-mediated immune response.

The present invention provides, in an additional aspect thereof, a method of producing a vaccine for protection against disease caused by respiratory syncytial virus (RSV) infection, comprising administering the immunogenic composition provided herein to a test host to determine the amount of and frequency of administration thereof to confer protection against disease caused by a RSV; and formulating the immunogenic composition in a form suitable for administration to a treated host in accordance with the determined amount and frequency of administration. The treated host may be a human.

A further aspect of the invention provides a method of determining the presence in a sample of antibodies specifically reactive with an F, G or M protein of respiratory syncytial virus (RSV), comprising the steps of:

(a) contacting the sample with the mixture as provided herein to produce complexes comprising a respiratory syncytial virus protein and any said antibodies present in the sample specifically reactive therewith; and

(b) determining production of the complexes.

In a further aspect of the invention, there is provided a method of determining the presence in a sample of a F, G or M protein of respiratory syncytial virus (RSV) comprising the steps of:

(a) immunizing a subject with the immunogenic composition as provided herein, to produce antibodies specific for the F, G and M proteins of RSV;

(b) contacting the sample with the antibodies to produce complexes comprising any RSV protein present in the sample and the protein specific antibodies; and

(c) determining production of the complexes.

A further aspect of the invention provides a diagnostic kit for determining the presence of antibodies in a sample specifically reactive with a F, G or M protein of respiratory syncytial virus, comprising:

(a) a mixture as provided herein;

(b) means for contacting the mixture with the sample to produce complexes comprising a respiratory syncytial virus protein and any said antibodies present in the sample; and

(c) means for determining production of the complexes.

In an additional aspect of the invention, there is provided a method of producing monoclonal antibodies specific for F, G or M proteins of respiratory syncytial virus (RSV), comprising:

(a) administrating an immunogenic composition as provided herein to at least one mouse to produce at least one immunized mouse,

(b) removing B-lymphocytes from the at least one immunized mouse;

(c) fusing the B-lymphocytes from the at least one immunized mouse with myeloma cells, thereby producing hybridomas;

(d) cloning the hybridomas which produce a selected anti-RSV protein antibody;

(e) culturing the selected anti-RSV protein antibody-producing clones; and

(f) isolating anti-RSV protein antibodies from the selected cultures.

The present invention, in a further aspect, provides a method of producing a coisolated and copurified mixture of proteins of respiratory syncytial virus, which comprises growing RSV on cells in a culture medium, separating the grown virus from the culture medium, solubilizing at least the F, G and M proteins from the separated virus; and coisolating and copurifying the solubilized RSV proteins.

The coisolation and copurification may be effected by loading the solubilized proteins onto an ion-exchange matrix, preferably a calcium phosphate matrix, specifically a hydroxyapatite matrix, and selectively coeluting the F, G and M proteins from the ion-exchange matrix. The grown virus may first be washed with urea to remove contaminants without substantially removing F, G and M proteins.

Advantages of the present invention include:

- coisolated and copurified mixtures of F, G and M proteins of RSV;
- immunogenic compositions containing such proteins;
- procedures for isolating such protein; and

- diagnostic kits for identification of RSV and hosts infected thereby.

BRIEF DESCRIPTION OF DRAWINGS

Figure 1, containing panels a and b, shows SDS-PAGE analysis of a purified RSV A subunit preparation using acrylamide gels stained with silver, under both reduced (panel (a)) and non-reduced (panel (b)) conditions;

Figure 2, containing panels a, b, c and d, shows Western blot analysis of a purified RSV subunit preparation under reduced conditions;

Figure 3, containing panels a, b, c and d, shows Western blot analysis of a purified RSV subunit preparation under non-reduced conditions; and

Figure 4 shows SDS-PAGE analysis of a purified RSV B subunit preparation using acrylamide gels stained with silver under reduced conditions.

GENERAL DESCRIPTION OF INVENTION

As discussed above, the present invention provides the F, G and M proteins of RSV coisolated and copurified from RS virus. The virus is grown on a vaccine quality cell line, such as VERO cells and human diploid cells, such as MRC5 and WI38, and the grown virus is harvested. The fermentation may be effected in the presence of fetal bovine serum (FBS) and trypsin.

The viral harvest is filtered and then concentrated, typically using tangential flow ultrafiltration with a membrane of desired molecular weight cut-off, and diafiltered. The virus harvest concentrate may be centrifuged and the supernatant discarded. The pellet following centrifugation may first be washed with a buffer containing urea to remove soluble contaminants while leaving the F, G and M proteins substantially unaffected, and then recentrifuged. The pellet from the centrifugation then is detergent extracted to solubilize the F, G and M proteins from the pellet. Such detergent extraction may

be effected by resuspending the pellet to the original harvest concentrate volume in an extraction buffer containing a detergent, such as a non-ionic detergent, including TRITON® X-100, a non-ionic detergent which is octadienyl phenol (ethylene glycol)$_{10}$. Other detergents include octylglucoside and Mega detergents.

Following centrifugation to remove non-soluble proteins, the F, G and M protein extract is purified by chromatographic procedures. The extract may first be applied to an ion exchange chromatography matrix to permit binding of the F, G and M proteins to the matrix while impurities are permitted to flow through the column. The ion-exchange chromatography matrix may be any desired chromatography material, particularly a calcium phosphate matrix, specifically hydroxyapatite, although other materials, such as DEAE and TMAE and others, may be used.

The bound F, G and M proteins then are coeluted from the column by a suitable eluant. The resulting copurified F, G and M proteins may be further processed to increase the purity thereof.

The purified F, G and M proteins employed herein may be in the form of homo and hetero oligomers including F:G heterodimers and including dimers, tetramers and higher species. The RSV protein preparations prepared following this procedure demonstrated no evidence of any adventitious agent, hemadsorbing agent or live virus.

Groups of cotton rats were immunized intramuscularly with the preparations provided herein in combination with alum or Iscomatrix™ as adjuvant. Strong anti-fusion and neutralization titres were obtained, as shown in Tables 1 and 2 below. Complete protection against virus infection was obtained in the upper and lower respiratory tracts, as shown in Tables 3 and 4 below.

In addition, groups of mice were immunized intramuscularly with the preparation provided herein in combination with alum, Iscomatrix™, polyphosphazene and DC-chol as adjuvant. Strong neutralizing and anti-F antibody titres were obtained, as shown in Tables 5 and 6 below. In addition, complete protection against virus infection was obtained, as shown by the absence of virus in lung homogenates (Table 7 below).

Groups of monkeys also were immunized with the preparations provided herein in combination with alum or Iscomatrix™ as adjuvant. Strong neutralizing titres and anti-F antibody titres were obtained, as shown in Tables 8 and 9 below.

The animal immunization data generated herein demonstrate that, by employing mild detergent extraction of the major RSV proteins from virus and mild salt elution of the proteins from the ion-exchange matrix, there are obtained copurified mixtures of the F, G and M RSV proteins which are capable of eliciting an immune response in experimental animals models that confers protection against RSV challenge.

The invention extends to the mixture of F, G and M proteins from respiratory syncytial virus for use as a pharmaceutical substance as an active ingredient in a vaccine against disease caused by infection with respiratory syncytial virus.

In a further aspect, the invention provides the use of F, G and M proteins from respiratory syncytial virus for the preparation of a vaccinal composition for immunization against disease caused by infection with respiratory syncytial virus.

It is clearly apparent to one skilled in the art, that the various embodiments of the present invention have many applications in the fields of vaccination, diagnosis and treatment of respiratory syncytial virus infections, and the generation of immunological agents.

A further non-limiting discussion of such issue is further presented below.

1. Vaccine Preparation and Use

Immunogenic compositions, suitable to be used as vaccines, may be prepared from mixtures comprising immunogenic F, G and M proteins of RSV as disclosed herein. The immunogenic composition elicits an immune response which produces antibodies, including anti-RSV antibodies including anti-F, anti-G and anti-M antibodies. Such antibodies may be viral neutralizing and/or anti-fusion antibodies.

Immunogenic compositions including vaccines may be prepared as injectables, as liquid solutions, suspensions or emulsions. The active immunogenic ingredient or ingredients may be mixed with pharmaceutically acceptable excipients which are compatible therewith. Such excipients may include water, saline, dextrose, glycerol, ethanol, and combinations thereof. The immunogenic compositions and vaccines may further contain auxiliary substances, such as wetting or emulsifying agents, pH buffering agents, or adjuvants to enhance the effectiveness thereof. Immunogenic compositions and vaccines may be administered parenterally, by injection subcutaneous, intradermal or intramuscularly injection. Alternatively, the immunogenic compositions formed according to the present invention, may be formulated and delivered in a manner to evoke an immune response at mucosal surfaces. Thus, the immunogenic composition may be administered to mucosal surfaces by, for example, the nasal or oral (intragastric) routes. Alternatively, other modes of administration including suppositories and oral formulations may be desirable. For suppositories, binders and carriers may include, for example, polyalkalene glycols or triglycerides. Such suppositories may be formed from mixtures containing the

active immunogenic ingredient(s) in the range of about 0.5 to about 10%, preferably about 1 to 2%. Oral formulations may include normally employed carriers such as, pharmaceutical grades of saccharine, cellulose and magnesium carbonate. These compositions can take the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations or powders and contain about 1 to 95% of the active ingredient(s), preferably about 20 to about 75%.

The immunogenic preparations and vaccines are administered in a manner compatible with the dosage formulation, and in such amount as will be therapeutically effective, immunogenic and protective. The quantity to be administered depends on the subject to be treated, including, for example, the capacity of the individual's immune system to synthesize antibodies, and if needed, to produce a cell-mediated immune response. Precise amounts of active ingredient required to be administered depend on the judgment of the practitioner. However, suitable dosage ranges are readily determinable by one skilled in the art and may be of the order of micrograms to milligrams of the active ingredient(s) per vaccination. Suitable regimes for initial administration and booster doses are also variable, but may include an initial administration followed by subsequent booster administrations. The dosage may also depend on the route of administration and will vary according to the size of the host.

The concentration of the active ingredient protein in an immunogenic composition according to the invention is in general about 1 to 95%. A vaccine which contains antigenic material of only one pathogen is a monovalent vaccine. Vaccines which contain antigenic material of several pathogens are combined vaccines and also belong to the present invention. Such combined vaccines contain, for example, material from various pathogens or

from various strains of the same pathogen, or from combinations of various pathogens. In the present invention, as noted above, F, G and M proteins of RSV A and RSV B are combined in a single multivalent immunogenic composition which also may contain other immunogens.

Immunogenicity can be significantly improved if the antigens are co-administered with adjuvants. Adjuvants enhance the immunogenicity of an antigen but are not necessarily immunogenic themselves. Adjuvants may act by retaining the antigen locally near the site of administration to produce a depot effect facilitating a slow, sustained release of antigen to cells of the immune system. Adjuvants can also attract cells of the immune system to an antigen depot and stimulate such cells to elicit immune responses.

Immunostimulatory agents or adjuvants have been used for many years to improve the host immune responses to, for example, vaccines. Intrinsic adjuvants, such as lipopolysaccharides, normally are the components of the killed or attenuated bacteria used as vaccines. Extrinsic adjuvants are immunomodulators which are formulated to enhance the host immune responses. Thus, adjuvants have been identified that enhance the immune response to antigens delivered parenterally. Some of these adjuvants are toxic, however, and can cause undesirable side-effects, making them unsuitable for use in humans and many animals. Indeed, only aluminum hydroxide and aluminum phosphate (collectively commonly referred to as alum) are routinely used as adjuvants in human and veterinary vaccines. The efficacy of alum in increasing antibody responses to diphtheria and tetanus toxoids is well established and a HBsAg vaccine has been adjuvanted with alum. While the usefulness of alum is well established for some applications, it has limitations. For example, alum is ineffective for

influenza vaccination and usually does not elicit a cell mediated immune response. The antibodies elicited by alum-adjuvanted antigens are mainly of the IgG1 isotype in the mouse, which may not be optimal for protection by some vaccinal agents.

A wide range of extrinsic adjuvants can provoke potent immune responses to antigens. These include saponins complexed to membrane protein antigens (immune stimulating complexes), pluronic polymers with mineral oil, killed mycobacteria in mineral oil, Freund's incomplete adjuvant, bacterial products, such as muramyl dipeptide (MDP) and lipopolysaccharide (LPS), as well as lipid A, and liposomes.

To efficiently induce humoral immune responses (HIR) and cell-mediated immunity (CMI), immunogens are often emulsified in adjuvants. Many adjuvants are toxic, inducing granulomas, acute and chronic inflammations (Freund's complete adjuvant, FCA), cytolysis (saponins and Pluronic polymers) and pyrogenicity, arthritis and anterior uveitis (LPS and MDP). Although FCA is an excellent adjuvant and widely used in research, it is not licensed for use in human or veterinary vaccines because of its toxicity.

2. Immunoassays

The F, G and M proteins of RSV of the present invention are useful as immunogens for the generation of antibodies thereto, as antigens in immunoassays including enzyme-linked immunosorbent assays (ELISA), RIAs and other non-enzyme linked antibody binding assays or procedures known in the art for the detection of antibodies. In ELISA assays, the selected F, G or M protein or a mixture of proteins is immobilized onto a selected surface, for example, a surface capable of binding proteins such as the wells of a polystyrene microtiter plate. After washing to remove incompletely adsorbed material, a nonspecific protein, such as a

solution of bovine serum albumin (BSA) that is known to be antigenically neutral with regard to the test sample may be bound to the selected surface. This allows for blocking of nonspecific adsorption sites on the immobilizing surface and thus reduces the background caused by nonspecific binding of proteins in the antisera onto the surface.

The immobilizing surface is then contacted with a sample, such as clinical or biological materials, to be tested in a manner conducive to immune complex (antigen/antibody) formation. This may include diluting the sample with diluents, such as solutions of BSA, bovine gamma globulin (BGG) and/or phosphate buffered saline (PBS)/Tween. The sample is then allowed to incubate for from about 2 to 4 hours, at temperatures, such as of the order of about 25° to 37°C. Following incubation, the sample-contacted surface is washed to remove non-immunocomplexed material. The washing procedure may include washing with a solution, such as PBS/Tween or a borate buffer. Following formation of specific immunocomplexes between the test sample and the bound protein, and subsequent washing, the occurrence, and even amount, of immunocomplex formation may be determined by subjecting the immunocomplex to a second antibody having specificity for the first antibody. If the test sample is of human origin, the second antibody is an antibody having specificity for human immunoglobulins and in general IgG. To provide detecting means, the second antibody may have an associated activity such as an enzymatic activity that will generate, for example, a color development upon incubating with an appropriate chromogenic substrate. Quantification may then be achieved by measuring the degree of color generation using, for example, a spectrophotometer.

EXAMPLES

The above disclosure generally describes the present invention. A more complete understanding can be obtained by reference to the following specific Examples. These Examples are described solely for purposes of illustration and are not intended to limit the scope of the invention. Changes in form and substitution of equivalents are contemplated as circumstances may suggest or render expedient. Although specific terms have been employed herein, such terms are intended in a descriptive sense and not for purposes of limitation.

Methods of determining tissue culture infectious $dose_{50}$ ($TCID_{50}$/mL), plaque and neutralization titres, not explicitly described in this disclosure are amply reported in the scientific literature and well within the scope of those skilled in the art. Protein concentrations were determined by the bicinchoninic acid (BCA) method as described in the Pierce Manual (23220, 23225; Pierce Chemical company, U.S.A.), incorporated herein by reference.

CMRL 1969 and Iscove's Modified Dulbecco's Medium (IMDM) culture media were used for cell culture and virus growth. The cells used in this study are vaccine quality African green monkey kidney cells (VERO lot M6) obtained from Institut Mérieux. The RS viruses used were the RS virus subtype A (Long and A2 strains) obtained from the American Type culture Collection (ATCC), a recent subtype A clinical isolate and RSV subtype B clinical isolate from Baylor College of Medicine.

Example 1:

This Example illustrates the production of RSV on a mammalian cell line on microcarrier beads in a 150 L controlled fermenter.

Vaccine quality African green monkey kidney cells (VERO) at a concentration of 10^5 cells/mL were added to 60 L of CMRL 1969 medium, pH 7.2 in a 150 L bioreactor containing 360 g of Cytodex-1 microcarrier beads and stirred for 2 hours. An additional 60 L of CMRL 1969 was added to give a total volume of 120 L. Fetal bovine serum was added to achieve a final concentration of 3.5%. Glucose was added to a final concentration of 3 g/L and L-glutamine was added to a final concentration of 0.6 g/L. Dissolved oxygen (40%), pH (7.2), agitation (36 rpm), and temperature (37°C) were controlled. Cell growth, glucose, lactate, and glutamine levels were monitored. At day 4, the culture medium was drained from the fermenter and 100 L of E199 media (no fetal bovine serum) was added and stirred for 10 minutes. The fermentor was drained and filled again with 120 L of E199.

An RSV inoculum of RSV subtype A was added at a multiplicity of infection (M.O.I.) of 0.001 and the culture was then maintained for 3 days before one-third to one-half of the medium was drained and replaced with fresh medium. On day 6 post-infection, the stirring was stopped and the beads allowed to settle. The viral culture fluid was drained and filtered through a 20 µm filter followed by a 3 µm filter prior to further processing.

The clarified viral harvest was concentrated 75- to 150-fold using tangential flow ultrafiltration with 300 NMWL membranes and diafiltered with phosphate buffered saline containing 10% glycerol. The viral concentrate was stored frozen at -70°C prior to further purification.

Example 2:

This Example illustrates the process of purifying RSV subunit from a viral concentrate of RSV subtype A.

A solution of 50% polyethylene glycol-8000 was added to an aliquot of virus concentrate prepared as described in Example 1 to give a final concentration of 6%. After stirring at room temperature for one hour, the mixture was centrifuged at 15,000 RPM for 30 min in a Sorvall SS-34 rotor at 4°C. The viral pellet was suspended in 1 mM sodium phosphate, pH 6.8, 2 M urea, 0.15 M NaCl, stirred for 1 hour at room temperature, and then recentrifuged at 15,000 RPM for 30 min. in a Sorvall SS-34 rotor at 4°C. The viral pellet was then suspended in 1 mM sodium phosphate, pH 6.8, 50 mM NaCl, 1% Triton X-100 and stirred for 30 minutes at room temperature. The insoluble virus core was removed by centrifugation at 15,000 RPM for 30 min. in a Sorval SS-34 rotor at 4°C. The soluble protein supernatant was applied to a column of ceramic hydroxyapatite (type II, Bio-Rad Laboratories) and the column was then washed with five column volumes of 1 mM sodium phosphate, pH 6.8, 50 mM NaCl, 0.02% Triton X-100. The RSV subunit composition from RSV subtype A, containing the F, G and M proteins, was obtained by eluting the column with 10 column volumes of 1 mM sodium phosphate, pH 6.8, 400 mM NaCl, 0.02% Triton X-100.

Example 3:

This Example illustrates the analysis of RSV subunit preparation obtained from RSV subtype A by SDS polyacrylamide gel electrophoresis (SDS-PAGE) and by immunoblotting.

The RSV subunit composition prepared as described in Example 2 was analyzed by SDS-PAGE using 12.5% acrylamide gels. Samples were electrophoresed in the presence or absence of 2-mercaptoethanol (reducing agent). Gels were stained with silver stain to detect the viral proteins (Figure 1, panels a and b). Immunoblots of replicate gels were prepared and probed with a mouse monoclonal antibody (mAb 5353C75) to F

glycoprotein (Figures 2, panel a and 3, panel a), or a mouse monoclonal antibody (mAb 131-2G), to G glycoprotein (Figures 2, panel b and 3, panel b) or guinea pig anti-serum (gpl78) against an RSV M peptide (peptide sequence: LKSKNMLTTVKDLTMKTLNPTHDIIALCEFEN - SEQ ID No:1) (Figures 2, panel c and 3, panel c), or goat antiserum (Virostat #0605) against whole RSV (Figures 2, panel d and 3, panel d). Densitometric analysis of the silver-stained gel of the RSV subunit preparation electrophored under reducing conditions indicated a compositional distribution as follows:

G glycoprotein (95 kDa form) = 10%

F_1 glycoprotein (48 kDa) = 30%

M protein (31 kDa) = 23%

F_2 glycoprotein (23 kDa) = 19%

The F glycoprotein migrates under non-reducing conditions as a heterodimer of approximately 70 kDa (F_0) as well as higher oligomeric forms (dimers and trimers) (Figure 3, panel a).

Example 4:

This Example illustrates the immunogenicity of the RSV subunit preparation in cotton rats.

Groups of five cotton rats were immunized intramuscularly (0.1 mL on days 0 and 28 with 1 µg or 10 µg the RSV subunit preparation, produced as described in Example 2 and formulated with either 1.5 mg/dose alum or 5 µg/dose Iscomatrix™ (Iscotec, Sweden). Blood samples were obtained on day 41 and assayed for anti-fusion titres and neutralization titres. The rats were challenged intranasally on day 43 with RSV and sacrificed four days later. Lavages of the lungs and naso pharynx were collected and assayed for RSV titres. Strong anti-fusion and neutralizing antibody titres were induced as shown in Tables 1 and 2 below. In addition, complete protection against virus infection was obtained with the exception of one rat, in both the

upper and lower respiratory tracts (Tables 3 and 4 below).

Example 5:

This Example illustrates the immunogenicity of the RSV subunit preparation in mice.

Groups of six BALB/c mice were immunized intramuscularly (0.1 mL) on days 0 and 28 with various doses of the RSV subunit preparation, produced as described in Example 2 and formulated with either 1.5 mg/dose alum, 10 µg/dose Iscomatrix™, 200 µg/dose polyphosphazene (PCPP) or 200 µg/dose DC-chol. The various preparations tested are set forth in Tables 5, 6 and 7 below. Blood samples were obtained on days 28 and 42 and assayed for neutralizing antibody titres and anti-F antibody titres. The mice were challenged on day 44 with RSV and sacrificed four days later. Lungs were removed and homogenized to determine virus titres. Strong neutralization titres and anti-F antibody titres were elicited as shown in Tables 5 and 6 below. In addition, complete protection against virus infection was obtained as shown by the absence of virus in lung homogenates and nasal washes (Table 7 below).

Example 6:

This Example illustrates the immunogenicity of RSV subunit preparation in African green monkeys.

Groups of four monkeys were immunized intramuscularly (0.5 mL on days 0 and 21 with 100 µg of the RSV subunit preparation, produced as described in Example 2 and formulated with either 1.5 mg/dose alum or 50 µg/dose Iscomatrix™. Blood samples were obtained on days 21, 35 and 49 and assayed for neutralizing and anti-F antibody titres. Strong neutralizing and anti-F antibody titres were obtained as shown in Tables 8 and 9 below.

Example 7:

This Example further illustrates the production of RSV or a mammalin cell live or microbeads in a 150L controlled fermenter.

Vaccine quality African green monkey kidney cells (Vero cells) were added to 150L of Iscove's Modified Dulbecco's Medium (IMDM) containing 3.5% fetal bovine serum, pH 7.2, to a final concentration of 2 x 10^5 cells/mL (range 1.5 to 3.5 cells/mL), in a 150 L bioreactor containing 450 g of Cytodex-1 microcarrier beads (3 g/L). Following cell inoculation, dissolved oxygen (40 percent air saturation (range 25 to 40%), pH (7.1 ± 0.2)), agitation (36 ± 2 rpm), and temperature (37° ± 0.5°C) were controlled. Initial cell attachment to beads, cell growth (cell number determination), and growth medium levels of glucose and lactate were monitored on a daily basis. Infection of the Vero cell culture occurred three to four days following initiation of cell growth, when the concentration of cells was in the range 1.5 to 2.0 x 10^6 cells/mL. Agitation was stopped and the microcarrier beads were allowed to settle for 60 minutes and the culture medium was drained from the bioreactor using a drain line placed approximately 3 cm above the settled bead volume. Seventy-five L of IMDM without fetal bovine serum (wash medium) was added and the mixture stirred at 36 rpm for 10 minutes. The agitation was stopped and the microcarrier beads allowed to settle for 30 minutes. The wash medium was removed using the drain line and then the bioreactor was filled to 75 L (half volume) with IMDM without fetal bovine serum.

For infection, an RSV inoculum of RSV subtype B was added at a multiplicity of infection (M.O.I.) of 0.001 and virus adsorption to cells at half volume was carried out for 2 hours with stirring at 36 rpm. Seventy-five L of IMDM was then added to the bioreactor to a final

volume of 150 L. Following infection, dissolved oxygen (40 percent air saturation (range 10 - 40%)), pH (7.25 ± 0.1), agitation (36 ± 2 rpm) and temperature (37° ± 0.5°C) were controlled. Following infection, cell growth (cell number determination) medium, glucose and lactate levels, RSV F and G antigens and RSV infectivity were monitored on a daily basis. On day 3 following infection, agitation was stopped, the microcarrier beads were allowed to settle for 60 minutes, and 75 L (50%) of the medium was removed via the drain line and replaced with fresh medium. Eight days (range seven to nine days) following infection, when complete virus-induced cytopathic effect was observed (i.e. cells were detached from the microcarrier beads, and oxygen was no longer being consumed by the culture), the agitator was stopped and the microcarrier beads were allowed to settle for 60 minutes. The virus containing culture fluid was removed from the bioreactor and transferred to a holding vessel. Seventy-five L of IMDM without fetal bovine serum was added to the bioreactor and agitated at 75 rpm for 30 minutes. The microcarrier beads were allowed to settle for 30 minutes, the rinse fluid was removed from the bioreactor and combined with the harvested material in the holding vessel.

The harvested material was concentrated approximately 20-fold by tangential flow filtration (i.e. virus-containing material was retained by the membrane) using a 500 or 1000 kilodalton (K) ultrafiltration membrane or alternatively a 0.45 μM microfiltration membrane to a final volume of 10L. The concentrated material was diafiltered with 10 volumes of phosphate-buffered saline, pH 7.2. The diafiltered viral concentrate was stored frozen at -70°C prior to further purification.

Example 8:

This Example illustrates the process of purifying RSV subunit from a viral concentrate of RSV subtype B.

A virus concentrate, prepared as described in Example 7, was centrifuged at 15,000 rpm for 30 min in a Sorvall SS-34 rotor at 4°C. The viral pellet was then suspended in 1 mM sodium phosphate, pH 6.8, 300 mM NaCl, 2% Triton X-100 and stirred for 30 minutes at room temperature. The insoluble virus core was removed by centrifugation at 15,000 RPM for 30 min in a Sorval SS-34 rotor at 4°C. The soluble protein supernatant was applied to a column of ceramic hydroxyapatite (type I, Bio-Rad Laboratories) and the column was then washed with ten column volumns of 1 mM sodium phosphate, pH 6.8, 10 mM NaCl, 0.02% Triton X-100. The RSV subunit composition, containing the F, G and M protein, was obtained by eluting the column with 10 column volumes of 1 mM sodium phosphate, pH 6.8, 600 mM NaCl, 0.02% Triton X-100. In some instances, the RSV subunit composition was further purified by first diluting the eluate from the first ceramic hydroxyapatite column to lower the NaCl concentration to 400 mM NaCl and then applying the diluted subunit onto a column of ceramic hydroxyapatite (type II, Bio-Rad Laboratories). The flowthrough from this column is the purified RSV subunit composition from RSV subtype B.

Example 9:

This Example illustrates the analysis of RSV subunit preparation obtained from RSV subtype B by SDS polyacryamide gel electrophoresis (SDS-PAGE).

The RSV subunit composition prepared as described in Example 8 was analyzed by SDS-PAGE using a 15.0% acrylamide gel. The sample was electrophoresed in the presence of 2-mercaptoethanol (reducing agent). The gel was stained with silver stain to detect the viral proteins (Figure 4). Densitometric analysis of the

silver-stained gel of the RSV subunit preparation under reducing conditions indicated a compositional distribution of the proteins as follows:

G glycoprotein (95 kDa form) = 21%

F_1 glycoprotein (48 kDa) = 19%

M protein (31 kDa) = 22%

F_2 glycoprotein (23 kDa) = 20%

SUMMARY OF DISCLOSURE

In summary of this disclosure, the present invention provides a coisolated and purified mixture of F, G and M proteins of RSV which is able to protect against RSV in relevant animal models of infection. Modifications are possible within the scope of this invention.

Table 1 - Serum Anti-Fusion Titres in Cotton Rats

Group	Mean titre (log_2)	Std. Dev (log_2)
Alum placebo	2.0	0.0
Iscomatrix™ placebo	2.3	0.5
RSV Subunit 1 µg with Alum	8.0	1.0
RSV Subunit 10 µg with Alum	7.5	1.0
RSV Subunit 1 µg with Iscomatrix™	10.4	1.3
RSV Subunit 10 µg with Iscomatrix™	10.0	1.6

Table 2 - Serum Neutralization Titres in Cotton Rats

Group	Mean titre (log_2)	Std. Dev. (log_2)
Alum placebo	2.0	0.0
Iscomatrix™ placebo	2.0	0.0
RSV Subunit 1 µg with Alum	9.6	1.3
RSV Subunit 10 µg with Alum	10.0	1.4
RSV Subunit 1 µg with Iscomatrix™	10.6	1.1
RSV Subunit 10 µg with Iscomatrix™	11.2	1.1

Table 3 - Pulmonary Wash RSV Titres in Cotton Rats

Group	Mean titre (log_{10}/g lung)	Std. Dev. (log_{10}/g lung)
Alum placebo	3.8	0.4
Iscomatrix™ placebo	3.7	0.5
RSV Subunit 1 µg with Alum	0.4	0.8
RSV Subunit 10 µg with Alum	0.0	0.0
RSV Subunit 1 µg with Iscomatrix™	0.0	0.0
RSV Subunit 10 µg with Iscomatrix™	0.0	0.0

Table 4 - Nasal Wash RSV Titres in Cotton Rats

Group	Mean titre (log_{10}/g lung)	Std. Dev. (log_{10}/g lung)
Alum placebo	3.2	0.5
Iscomatrix™ placebo	3.1	0.3
RSV Subunit 1 µg with Alum	0.0	0.0
RSV Subunit 10 µg with Alum	0.0	0.0
RSV Subunit 1 µg with Iscomatrix™	0.0	0.0
RSV Subunit 10 µg with Iscomatrix™	0.0	0.0

Table 5 - Serum Neutralization Titres in Balb/c Mice

Group	4 Week Bleed		6 Week Bleed	
	Mean titre (log_2)	Std. Dev. (log_2)	Mean titre (log_2)	Std. Dev. (log_2)
Alum placebo	3.0[1]	0.0	3.0	0.0
Iscomatrix™ placebo	3.0	0.0	3.0	0.0
PCPP placebo (200 µg)	ND	ND	3.0	0.0
DC-Chol placebo (200 µg)	ND	ND	3.0	0.0
RSV Subunit 0.1 µg with no adjuvant	ND	ND	3.0	0.0
RSV Subunit 0.1 µg with Alum	ND	ND	10.3	0.9
RSV Subunit 1 µg with Alum	6.5	0.6	8.7	1.0
RSV Subunit 10 µg with Alum	8.0	1.1	9.5	1.1
RSV Subunit 1 µg with Iscomatrix™	8.2	0.8	13.2	1.0
RSV Subunit 10 µg with Iscomatrix™	10.4	1.3	13.4	0.6
RSV Subunit 1 µg with PCPP (200 µg)	ND	ND	15.0	0.6
RSV Subunit 0.5 µg with DC-Chol (200 µg)	ND	ND	11.7	1.1

[1] minimal detectable titre in assay
ND = not determined

Table 6 - Serum Anti-F Titres in Balb/c Mice

Group	4 Week Bleed		6 Week Bleed	
	Mean titre (log_2titre/100)	Std. Dev. (log_2titre/100)	Mean titre (log_2titre/100)	Std. Dev. (log_2titre/100)
Alum placebo	0.5	1.2	0.0	0.0
Iscomatrix™ placebo	1.0	0.0	0.0	0.0
PCPP placebo (200 µg)	0.0	0.0	0.0	0.0
DC-Chol placebo (200 µg)	0.0	0.0	0.0	0.0
RSV Subunit 0.1 µg with no adjuvant	0.0	0.0	0.0	0.0
RSV Subunit 0.1 µg with Alum	7.0	1.0	12.4	0.9
RSV Subunit 1 µg with Alum	8.7	0.8	11.2	0.8
RSV Subunit 10 µg with Alum	9.7	0.8	12.3	1.0
RSV Subunit 1 µg with Iscomatrix™	8.5	0.6	13.3	0.5
RSV Subunit 10 µg with Iscomatrix™	10.0	0.0	13.0	0.0
RSV Subunit 1 µg with PCPP (200 µg)	10.2	0.8	14.0	0.7
RSV Subunit 0.5 µg with DC-Chol (200 µg)	9.7	1.4	13.0	1.0

Table 7 - Lung Virus Titres in Balb/c Mice

Group	Mean titre (log_{10}/g lung)	Std. Dev. (log_{10}/g lung)
Alum placebo	4.1	0.2
Iscomatrix™ placebo	3.5	0.1
PCPP placebo (200 µg)	5.2	0.2
DC-Chol placebo (200 µg)	5.0	0.3
RSV Subunit 0.1 µg with no adjuvant	5.3	0.1
RSV Subunit 0.1 µg with Alum	<1.7[1]	1.7
RSV Subunit 1 µg with Alum	<1.7	1.7
RSV Subunit 10 µg with Alum	<1.7	1.7
RSV Subunit 1 µg with Iscomatrix™	<1.7	1.7
RSV Subunit 10 µg with Iscomatrix™	<1.7	1.7
RSV Subunit 1 µg with PCPP (200 µg)	<1.7	1.7
RSV Subunit 0.5 µg with DC-Chol (200 µg)	<1.7	1.7

[1] minimal detectable virus titre in assay

Table 8 - Serum Neutralization Titres in African Green Monkeys

Group	3 Week Bleed		5 Week Bleed		7 Week Bleed	
	Mean titre (log_2)	Std. Dev. (log_2)	Mean titre (log_2)	Std. Dev. (log_2)	Mean titre (log_2)	Std. Dev. (log_2)
Alum placebo	3.3	0.0	3.3	0.0	3.3	0.0
Iscomatrix™ placebo	3.3	0.0	3.3	0.0	3.3	0.0
RSV Subunit 100 µg with Alum	11.3	1.3	14.6	1.3	11.5	1.4
RSV Subunit 100 µg with Iscomatrix™	10.8	0.7	15.1	0.1	11.9	0.5

Table 9 - Serum Anti-F Titres in African Green Monkeys

Group	3 Week Bleed		5 Week Bleed		7 Week Bleed	
	Mean titre (log_2 titre/100)	Std. Dev. (log_2 titre/100)	Mean titre (log_2 titre/100)	Std. Dev. (log_2 titre/100)	Mean titre (log_2 titre/100)	Std Dev (log_2 titre/100)
Alum placebo	0.0	0.0	0.0	0.0	0.0	0.0
Iscomatrix™ placebo	0.0	0.0	0.0	0.0	0.0	0.0
RSV Subunit 100 µg with Alum	6.5	1.9	9.3	1.0	9.0	1.2
RSV Subunit 100 µg with Iscomatrix™	5.5	1.0	9.8	0.5	9.5	1.0

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CLAIMS

What we claim is:

1. A mixture of purified fusion (F) protein, attachment (G) protein and matrix (M) protein of respiratory syncytial virus (RSV).

2. The mixture of claim 1 wherein said fusion (F) protein comprises multimeric fusion (F) proteins.

3. The mixture of claim 2 wherein, when analyzed under non-reducing conditions, said multimeric fusion (F) protein includes heterodimers of molecular weight approximately 70 kDa and dimeric and trimeric forms.

4. The mixture of claim 1 wherein, when analyzed under non-reducing conditions, said attachment (G) protein comprises G protein of molecular weight approximately 95 kDa and G protein of molecular weight approximately 55 kDa and oligomeric G protein.

5. The mixture of claim 1 wherein, when analyzed by SDS-PAGE under non-reducing conditions, said matrix (M) protein comprises M protein of molecular weight approximately 28 to 34 kDa.

6. The mixture of claim 1 wherein, when analyzed by reduced SDS-PAGE analysis, said fusion (F) protein comprises F_1 of molecular weight approximately 48 kDa and F_2 of molecular weight approximately 23 kDa, said attachment (G) protein comprises a G protein of molecular weight approximately 95 kDa and a G protein of molecular weight approximately 55 kDa, and said matrix (M) protein comprises an M protein of approximately 31 kDa.

7. The mixture of claim 1 wherein said F, G and M proteins are present in the relative proportions of:

F from about 35 to about 70 wt%

G from about 5 to about 30 wt%

M from about 10 to about 40 wt%

8. The mixture of claim 7 wherein, when analyzed by SDS-PAGE under reducing conditions and silver stained,

the ratio of F_1 of molecular weight approximately 48 kDa to F_2 of molecular weight approximately 23 kDa is between 1:1 to about 2:1 by scanning densitometry.

9. The mixture of claim 7 which is at least about 75% pure.

10. The mixture of claim 1 which is devoid of monoclonal antibodies.

11. The mixture of claim 1 which is devoid of lentil lectin and concanavalin A.

12. The mixture of claim 1 wherein said RSV proteins are non-denatured.

13. The mixture of claim 1 wherein said RSV proteins are from one or both of subtypes RSV A and RSV B.

14. A coisolated and copurified mixture of non-denatured proteins of respiratory syncytial virus (RSV), consisting essentially of the fusion (F) protein, attachment (G) protein and matrix (M) protein of RSV, wherein the mixture is free from lectins and is free from monoclonal antibodies.

15. An immunogenic composition comprising an immunoeffective amount of the mixture of claim 1.

16. The immunogenic composition of claim 15 formulated as a vaccine for *in vivo* administration to a host to confer protection against RSV.

17. The immunogenic composition of claim 15 further comprising at least one adjuvant or at least one immunomodulator.

18. The immunogenic composition of claim 17 wherein the at least one adjuvant is selected from the group consisting of aluminum phosphate, aluminum hydroxide, QS21, Quil A or derivatives or components thereof, calcium phosphate, calcium hydroxide, zinc hydroxide, a glycolipid analog, an octodecyl ester of an amino acid, a muramyl dipeptide, a lipoprotein, polyphosphazene, ISCOM matrix, DC-chol, DDA and bacterial toxins or derivatives thereof.

19. The immunogenic composition of claim 16 wherein the host is a primate.

20. The immunogenic composition of claim 19 wherein the primate is a human.

21. The immunogenic composition of claim 15 further comprising at least one additional immunogen.

22. The immunogenic composition of claim 21 wherein said at least one additional immunogen comprises at least one human parainfluenza virus (PIV) protein selected from the group consisting of PIV-1, PIV-2 and PIV-3.

23. A method of generating an immune response in a host, comprising administering thereto an immunoeffective amount of the immunogenic composition of claim 15.

24. The method of claim 23 wherein said immunogenic composition is formulated as a vaccine for *in vivo* administration to the host and said administration to the host confers protection against respiratory syncytial virus.

25. A method for producing a vaccine for protection against respiratory syncytial virus (RSV), comprising:

administering the immunogenic composition of claim 15 to a test host to determine the amount of and frequency of administration thereof to confer protection against disease caused by RSV; and

formulating the immunogenic composition in a form suitable for administration to a treated host in accordance with said determined amount and frequency of administration.

26. The method of claim 25 wherein the treated host is a human.

27. A method of producing monoclonal antibodies specific for fusion (F) protein, attachment (G) protein and matrix (M) protein of respiratory syncytial virus (RSV), comprising:

(a) administering an immunogenic composition of claim 15 to at least one mouse to produce at least one immunized mouse;

(b) removing B-lymphocytes from the at least one immunized mouse;

(c) fusing the B-lymphocytes from the at least one immunized mouse with myeloma cells, thereby producing hybridomas;

(d) cloning the hybridomas which produce a selected anti-RSV protein antibody;

(e) culturing the selected anti-RSV protein antibody-producing clones; and

(f) isolating anti-RSV protein antibodies from the selected cultures.

28. A method of producing a coisolated and copurified mixture of proteins of respiratory syncytial virus (RSV), which comprises:

growing RSV on cells in a culture medium;

separating the grown virus from the culture medium;

solubilizing at least the fusion (F) protein, attachment (G) protein and the matrix (M) protein from the separated virus; and

coisolating and copurifying the solubilized RSV proteins.

29. The method of claim 28 wherein said coisolation and copurification are effected by:

loading the solubilized proteins onto an ion-exchange matrix; and

selectively coeluting the F, G and M proteins from the ion-exchange matrix.

30. The method of claim 29 wherein said ion-exchange matrix is a hydroxyapatite matrix.

31. The method of claim 28 wherein said grown virus is washed with urea to remove contaminants without substantial removal of F, G and M proteins prior to solubilization step.

32. A method of determining the presence in a sample of antibodies specifically reactive with a fusion (F) protein, attachment (G) protein or matrix (M) protein of respiratory syncytial virus (RSV), comprising the steps of:

(a) contacting the sample with the mixture of claim 1 to produce complexes comprising a respiratory syncytial virus protein and any said antibodies present in the sample specifically reactive therewith; and

(b) determining production of the complexes.

33. A method of determining the presence in a sample of an F, G or M protein of respiratory syncytial virus, comprising the steps of:

(a) immunizing a subject with the immunogenic composition of claim 15 to produce antibodies specific for F, G and M proteins of RSV;

(b) contacting the sample with the antibodies to produce complexes comprising any RSV protein present in the sample and said protein specific antibodies; and

(c) determining production of complexes.

34. A diagnostic kit for determining the presence of antibodies in a sample specifically reactive with a fusion (F) protein, attachment (G) protein or a matrix (M) protein of respiratory syncytial virus comprising:

(a) a mixture of claim 1;

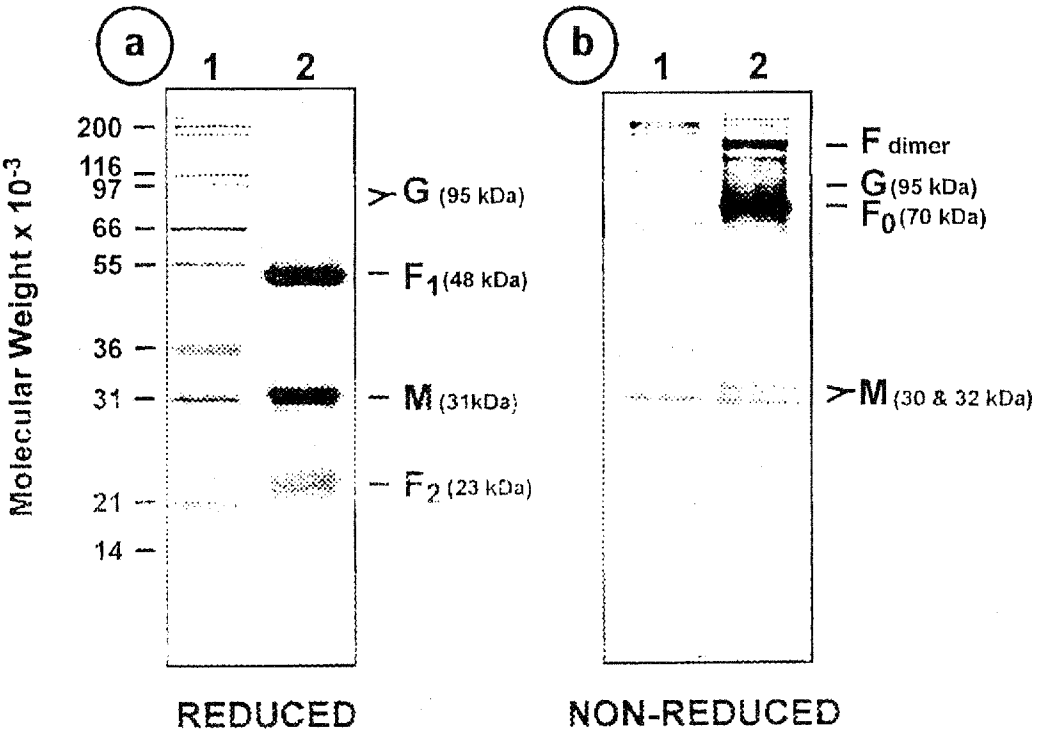
(b) means for contacting the immunogenic composition with the sample to produce complexes comprising a respiratory syncytial virus protein and any said antibodies present in the sample; and

(c) means for determining production of the complexes.

35. A mixture of purified fusion (F) protein, attachment (G) protein and matrix (M) protein of respiratory syncytial virus (RSV) for use as a pharmaceutical substance in a vaccine against disease caused by infection with respiratory syncytial virus.

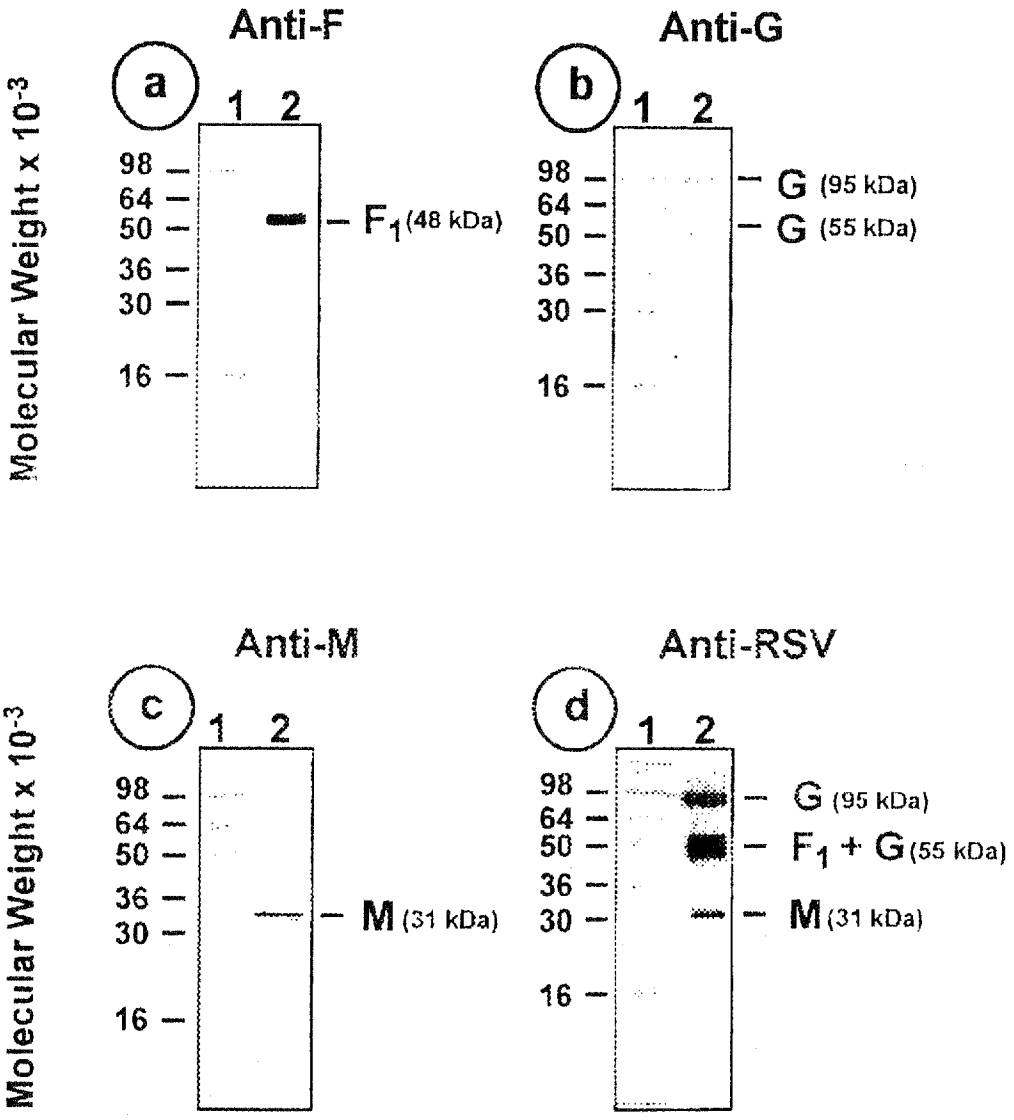
36. The use of a mixture of purified fusion (F) protein, attachment (G) protein and matrix (M) protein of respiratory syncytial virus for the preparation of a vaccinal composition for immunization against disease caused by infection with respiratory syncytial virus.

Figure 1 - SDS-PAGE Analysis of RSV Subunit (silver stain)



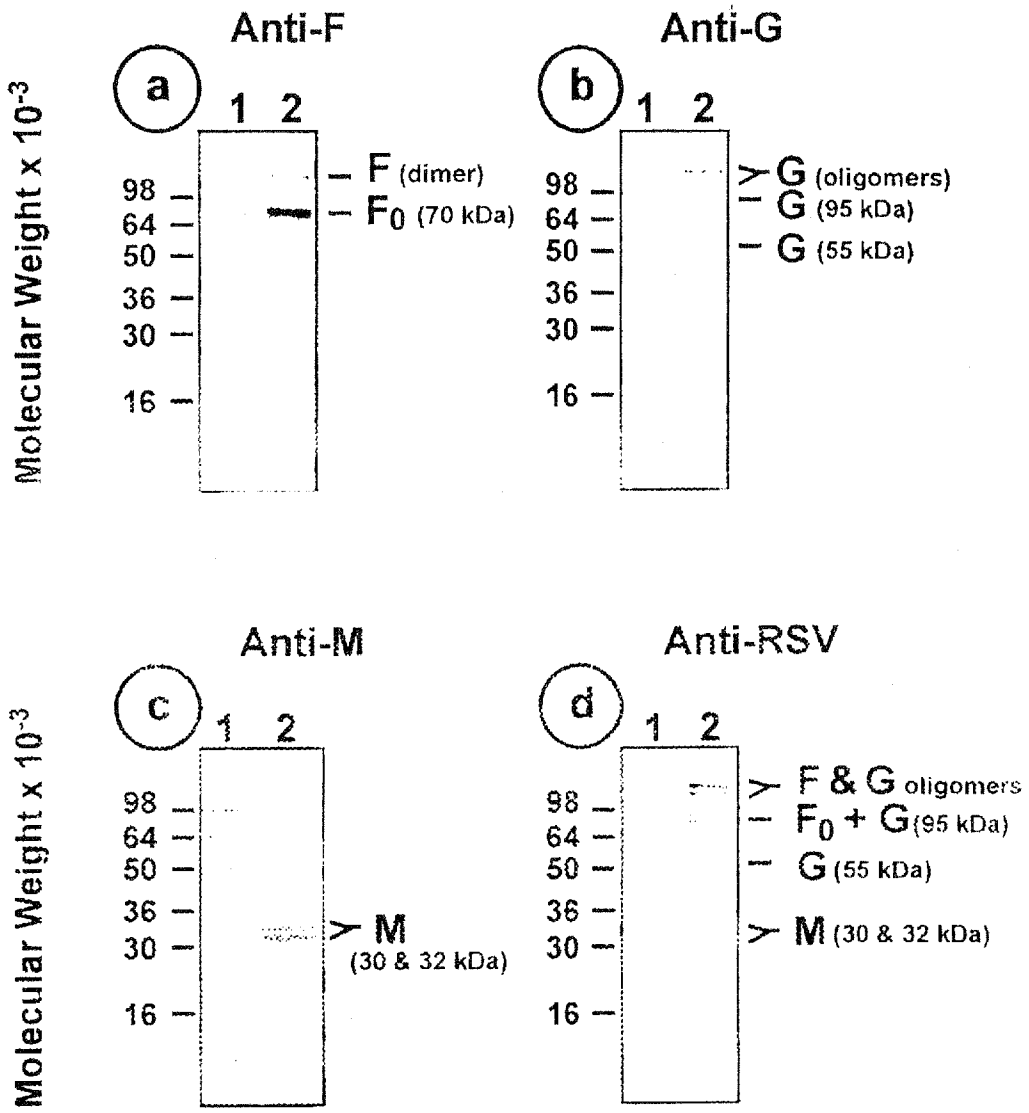
Lane 1 = Molecular Weight Standards
Lane 2 = RSV Subunit

Figure 2 - Western Blot Analysis of RSV Subunit Reduced



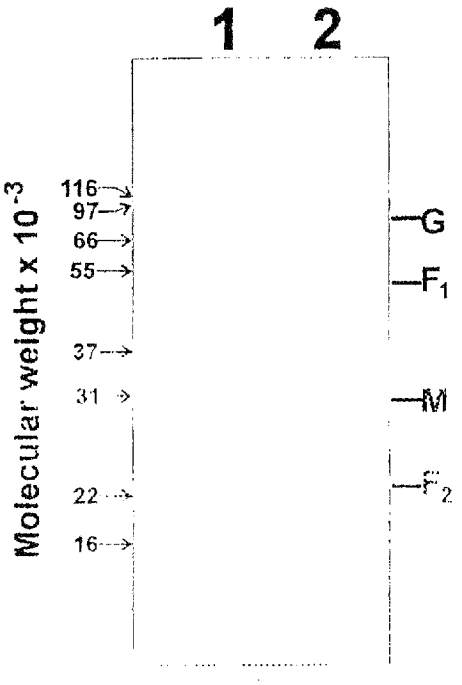
Lane 1 = Molecular Weight Standards
Lane 2 = RSV Subunit

Figure 3 - Western Blot Analysis of RSV Subunit Non-Reduced



Lane 1 = Molecular Weight Standards
Lane 2 = RSV Subunit

FIG. 4 - SDS-PAGE Analysis of RSV B Subunit (Silver Stain)



Lane 1= Molecular Weight Standard

Lane 2= RSV B Subunit

INTERNATIONAL SEARCH REPORT

Inte. ...nal Application No
PCT/CA 97/00497

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K14/135 A61K39/155 A61K39/39 C07K16/10 G01N33/569

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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Y	EWASYSHYN M.E. AND KLEIN M.H.: "Prospects for Immunization Against Respiratory Syncytial Virus" VACCINE RESEARCH, vol. 2, 1993, pages 263-272, XP002044888	1-13, 15-28, 32-36
A	(see especially page 266, lines 19-24) see the whole document	14,29-31

Y	WO 87 04185 A (UNIVERSITY OF NORTH CAROLINA) 16 July 1987 see claims 1-14	1-13, 15-27, 32-36

Y	WO 94 27636 A (AMERICAN CYANAMID CO.) 8 December 1994	17,18
A	see the whole document	1-16, 19-36

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[X] Further documents are listed in the continuation of box C

[X] Patent family members are listed in annex

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"A" document defining the general state of the art which is not considered to be of particular relevance

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1

Date of the actual completion of the international search	Date of mailing of the international search report
28 October 1997	19. 11. 97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Olsen, L

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INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/CA 97/00497

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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A	see the whole document	10,14, 28-31

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